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SUBCELLULAR LOCALIZATION OF BINDING SITES FOR CYTOCHALASIN D: EVIDENCE FROM ACTIVATION ENERGIES

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Summary

The activation energies for binding of tritiated cytochalasin D to HEp-2 cells and isolated plasma membrane were determined by Arrhenius plots. The higher value for intact cells (24 kcal/mol) compared to the plasma membrane fraction (4 kcal/mol at $> 11.5°C$, 18 kcal/mol at $< 11.5°C$) was taken as evidence that [^{3}H]cytochalasin D must penetrate the plasma membrane in order to reach its binding sites. The data support the conclusion that binding sites for [^{3}H]cytochalasin D are intracellular, on the cytoplasmic face of the plasma membrane (rather than within the lipid bilayer), and on microsomes (endomembranes).

We have previously reported [1] that both high and low affinity binding sites for cytochalasin D in HEp-2 cells are attached to the plasma membrane, while only low affinity sites are present on the endomembranes (i.e. microsomal fraction). More recently, we have obtained evidence that the binding sites on the plasma membrane are peripheral proteins (manuscript submitted for publication). Our data are consistent with identification of the high affinity sites for cytochalasin D as myosin: these sites do not bind the congeneric compound cytochalasin B and are distinct from those receptors for cytochalasin B which have been reported [2,3] to be part of the hexose transport system (manuscript submitted for publication). A subclass of the low affinity sites for cytochalasin D is also able to bind cytochalasin B; the low affinity sites associated with the plasma membrane may be actomyosin.

Because cytochalasins are lipophilic, it has been suggested that they are

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bound in the hydrophobic region of the phospholipid bilayer of the plasma membrane [e.g. 4–7]. We have presented results which do not support this hypothesis and furthermore provide evidence that binding sites for cytochalasin D are proteins exposed on the cytoplasmic, but not the external, face of the plasma membrane [1]. In this communication we present biophysical data corroborating the thesis that the receptors for cytochalasin D are intracellular.

HEp-2 cells were grown as monolayers on glass in Eagle's minimal essential medium supplemented with 10% newborn calf serum 60 units/ml penicillin and 60 μg/ml streptomycin (growth medium). Tritiated cytochalasin D dissolved in Me_2SO was obtained as described previously [1], and diluted appropriately with growth medium or buffer solution.

For experiments utilizing HEp-2 monolayers, cells planted in glass scintillation vials at a density of $2.5 \cdot 10^5$ cells/2 ml/vial were grown overnight before use. Suspended HEp-2 cells were prepared by trypsinization of HEp-2 monolayers grown in glass bottles; cells were washed and resuspended in (0.25 M sucrose/1 mM $MgCl_2$ /10 mM Tris, pH 7.0). The plasma membrane fraction was isolated from HEp-2 cells [8] and also suspended in sucrose/$MgCl_2$ /Tris buffer.

Samples and [^{3}H]cytochalasin D solutions were equilibrated in water baths at the appropriate temperatures for at least 15 min. Cell monolayers were then incubated at these temperatures with growth medium containing 0.26 μg/ml [^{3}H]cytochalasin D for appropriate time periods; cell-bound [^{3}H]cytochalasin D per monolayer was determined after this medium was aspirated and the monolayers were washed twice with chilled Earle's balanced saline solution. For cells or plasma membrane in sucrose/$MgCl_2$ /Tris buffer an equal volume of [^{3}H]cytochalasin D in sucrose/$MgCl_2$ /Tris buffer was added (final concentration, 0.52 μg/ml [^{3}H]cytochalasin D). The tubes were incubated at appropriate temperatures for the desired time intervals and then rapidly chilled in an ice bath for about 15 s before determination of bound [^{3}H]cytochalasin D by centrifugation (7000 × *g*/10 min/0 to 4°C) and washing of the pellets in chilled sucrose/$MgCl_2$ /Tris buffer. Washed pellets were suspended in distilled water; samples were taken for measurement of protein [9] and tritium. The washed monolayers and samples of pellets were solubilized in soluene-100 (Packard Instrument Co.) and counted in an Omnifluortoluene (4 g/l) scintillation cocktail (New England Nuclear). Efficiency of counting was determined by internal standardization. Rates of binding were expressed as dpm [^{3}H]cytochalasin D/μg protein/min for HEp-2 cells and membrane in sucrose/$MgCl_2$ /Tris buffer and as arbitrary units [^{3}H]cytochalasin D/monolayer/15 min for HEp-2 monolayers (see Fig. 1). Activation energies were calculated from the slopes of the Arrhenius plots shown in Fig. 1.

Rates of binding of [^{3}H]cytochalasin D to HEp-2 cells in monolayer at various temperatures, obtained from curves of uptake vs. time, were graphed in an Arrhenius plot (Fig. 1). The resulting line drawn through these points showed no evidence of discontinuity over the temperature range examined (0° to 37°C). From the slope of the line an activation energy of 24 kcal/mol was calculated.

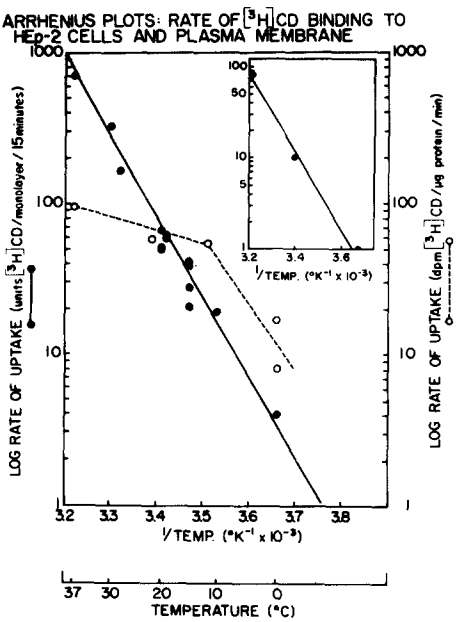

Fig. 1. Arrhenius Plots. Rate of binding of [^{3}H]cytochalasin D at various temperatures were plotted versus the inverse of the temperature in °K (X 10^3): (●) HEp-2 monolayers: (○) HEp-2 plasma membrane; Inset: HEp-2 cells (●) suspended in sucrose/$MgCl_2$ /Tris buffer (note change of scale; units are those of right ordinate). Left ordinate refers to monolayer samples, right ordinate to plasma membrane. Binding to monolayers is expressed in arbitrary units.

Since the binding assay for isolated plasma membrane suspended in (0.25 M sucrose/1 mM $MgCl_2$ /10 mM Tris-HCl pH 7.0) employed a centrifugation technique (see above), we tested the validity of comparing an activation energy obtained by this method with one based on the monolayer assay by also determining the value for intact HEp-2 cells suspended in sucrose/$MgCl_2$ /Tris buffer. The rates of binding of [^{3}H]cytochalasin D to cells in sucrose/$MgCl_2$ /Tris buffer at three temperatures gave a linear Arrhenius plot (Fig. 1 inset) and an activation energy of 20 kcal/mol. Since both methods gave similar values for whole cells, it seemed valid to compare them. The rates of binding of [^{3}H]cytochalasin D to isolated HEp-2 plasma membrane in sucrose/$MgCl_2$ /Tris buffer were determined at various temperatures. The resulting Arrhenius plot (Fig. 1) appeared to have a discontinuity at some temperature below about 11°C. Although it was difficult to measure precisely the low rate of binding at 0°C, the two values obtained (8 and 17 dpm/µg protein/min) evidently did not fall on the line determined by points at the higher temperatures. The activation energy calculated for the temperature range of 11.5° to 37°C was 4 kcal/mol. The line sketched in Fig. 1 for temperatures below 11.5°C yielded an activation energy of 18 kcal/mol. This latter value may be an underestimate since more points in the range 0 to 10°C would be needed to determine the exact position of the discontinuity.

From Arrhenius plots we have calculated an apparent activation energy of 24 kcal/mol for cells and, for isolated plasma membrane, the values of 4 kcal/mol (at 11.5° to 37°C) and approximately 18 kcal/mol (at 0° to

11.5°C). The activation energy represents the energy barrier which must be overcome in the slowest (i.e. rate determining) step of a process. For binding of [^{3}H]cytochalasin D to the intact cell, several "reaction pathways" in the binding process can be envisaged:

Case A. If the binding site is on the outside of the cell, in the aqueous environment, [^{3}H]cytochalasin D can associate immediately.

Case B. If the binding site is within the membrane lipid bilayer, [^{3}H]-cytochalasin D must leave the aqueous phase and penetrate into the lipid bilayer.

Case C. If the binding site is inside the cell (e.g. on the cytoplasmic side of the plasma membrane and/or on an internal organelle such as the endomembranes that comprise the microsomal fraction), [^{3}H]cytochalasin D must penetrate into the membrane, diffuse within the lipid bilayer, cross the lipid-cytoplasmic interface, enter the cytoplasm of the cell, and possibly diffuse within the cytoplasm until it reaches a binding site with which it associates.

In Case A, either the cell or isolated membrane would have its binding sites directly available to [^{3}H]cytochalsin D in the aqueous medium and thus the binding process would be equivalent. Case B requires that [^{3}H]cytochalsin D penetrate the lipid-water interface to reach its binding sites regardless of whether one or both sides of the plasma membrane are in direct contact with the external medium, again with equivalent pathways and activation energies for binding to either the cell or the isolated plasma membrane. In Case C, binding of [^{3}H]cytochalasin D to the isolated plasma membrane bypasses all but the last step (association with a cytochalasin D binding site) in the pathway described for binding to the intact cell. Thus, unless the rate determining step in binding to cells is the association process, the activation energy for binding to isolated plasma membrane must be lower than the value for intact cells.

Since the activation energy measured for binding of [^{3}H]cytochalasin D to intact cells was greater than the values for plasma membrane, only Case C can apply to the binding process in cells. Therefore, [^{3}H]cytochalasin D permeates the plasma membrane and binds at an intracellular location. In fact, the observed activation energy of 24 kcal/mol in the range reported for the permeation of various substances through cell membranes [cf. page 33 in ref. 10]. It has been asserted that the organization (i.e. mutual relationship) of components may be disrupted in the isolated plasma membrane as compared with the plasma membrane of the intact cell [11], and it might be argued that a lower activation energy for binding to the isolated membrane could result from such disarrangement. This explanation seems very improbable, because the binding activity of the isolated membrane is similar in rate, reversibility, and affinity to the binding observed in whole cells [5,12]. In addition, there is no a priori reason to assume that a rearrangement of membrane components would decrease the energy of activation (see, e.g. ref. 13). These data therefore indicate that [^{3}H]cytochalasin D bound by the cell is located intracellularly.

Because HEp-2 cells possess more than one class of binding sites [1], it is possible that the high activation energy obtained for intact cells is representative of only one class of sites whose response to temperature

obscured any effects on the other class(es). In this case, the argument outlined above might apply to microsomal binding sites in the whole cell, which are necessarily intracellular, regardless of whether plasma membrane binding sites were on or within the lipid bilayer of the cell. However, the inability of proteolysis of the inact cell to decrease binding activity for [^{3}H]cytochalsin D [1] suggests that binding sites are not located on the extracellular side of the plasma membrane. Furthermore, the low activation energy of 4 kcal/mol obtained for binding of [^{3}H]cytochalasin to isolated plasma membrane (above 11.5°C) is in the range found for diffusion of a variety of solutes in water (4 to 5 kcal/mol; see ref. 10); penetration of the lipid bilayer, which would be required for binding of [^{3}H]cytochalsin D in the hydrocarbon region of the membrane, should exhibit a higher activation energy [10]. Therefore, this low activation energy suggests that binding sites of the isolated plasma membrane are not located within the lipid bilayer, but rather are exposed to the aqueous medium and thus are displayed on the cytoplasmic face of the plasma membrane.

The discontinuity of the Arrhenius plot for membranes which occurs at about 11.5° C may be ascribed to some indirect effect on the binding sites by a phase transition or separation of membrane phospholipids under the influence of a change in temperature (refs 15—17, inter alia; review in ref. 14); or it could reflect a conformational change in the binding site itself, because proteins may also undergo thermally-induced alterations in structure [14]. The lack of a similar discontinuity in the Arrhenius plot for intact cells is explained by the observation that the activation energy for binding of [^{3}H]-cytochalasin D is higher than the values for the plasma membrane, implying that a different step (with a higher energy barrier) is rate-limiting for the cell as compared to the isolated membrane. Since the activation energy depends only on the rate limiting step, the behavior of the "temperature-sensitive" step, which is rate-limiting for binding of [^{3}H]cytochalasin D to the isolated plasma membrane, but not for binding to the whole cell, does not affect the value obtained for the activation energy of the intact cell.

In summary, the results presented here support our earlier conclusion [1] that receptors for [^{3}H]cytochalasin D are intracellular. Similarly, it has been reported that binding sites for the congeneric compound, cytochalasin B, are not exposed on the surface of human red blood cells [2] and are present on microsomes and within, or on the inner face of the plasma membrane of Ehrlich-Lettre ascites carcinoma cells [6]. Furthermore, our data suggest that the binding sites for [^{3}H]cytochalasin D on the cytoplasmic face of the plasma membrane are not buried within the lipid bilayer, but rather are exposed to the (aqueous) cytoplasm. This latter observation is compatible with our finding that the receptors are most likely peripheral proteins of the membrane located within the subplasmalemmal actomyosin contractile network of the cell (manuscript submitted for publication).

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